THE UNIVERSITY OF ALBERTA

A HIGH MOLECULAR WEIGHT VASOACTIVE COMPOUND IN THE SALIVARY GLAND

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled: "A High Molecular Weight Vasoactive Compound In The Salivary Gland", submitted by Walter J. Leonard in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The literature relating to hypo- and hypertensive substances in saliva and salivary glands is reviewed, with particular reference to kallikrein and sialotonin.

A method is described for the separation of kallikrein and sialotonin from saliva collected from a submaxillary gland of a cat during parasympathetic nerve stimulation. Attempts to make a stable preparation of sialotonin were unsuccessful.

The effect of sialotonin on regional blood flows was investigated: sialotonin causes a brief but marked vasoconstriction in the submaxillary gland. It is suggested that this could be used as an assay system for sialotonin in preference to the more unspecific measurement of pressor effects. Sialotonin also causes vasoconstriction in the blood vessels of the small intestine but is without effect on the blood vessels of the hind limb.

ACKNOWLEDGMENT

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Chapter I

INTRODUCTION

1. Hypotensive substances in saliva and salivary glands

a) Historical survey

Secker (1934a,b) demonstrated the marked depressor effect of intravenous injection of saliva secreted from the submaxillary gland of the cat during parasympathetic or sympathetic nerve stimulation. Since the fall in blood pressure was potentiated by eserine and abolished by atropine, Secker concluded that it was due to an acetylcholine-like substance which was liberated at the secretory nerve terminals and washed out from the gland in the saliva.

The next year, Gibbs (1935) reported results which conflicted with those of Secker: although atropine blocked an acetylcholine-induced fall in blood pressure it did not affect that due to the intravenous injection of saliva. Larson (1935) confirmed that saliva has hypotensive properties, but concluded that the active constitutent was not acetylcholine, since protein-free ultrafiltrates of saliva were without effect on the blood pressure. Although Larson did not test the protein residue, he suggested that the depressor activity of saliva was due to a substance of high molecular weight, or to one which was combined with a large molecule in saliva.

Experiments to identify the salivary depressor substance were extended by Feldberg and Guimarais (1935a,b).

They concluded that the active substance was not acetylcholine, choline, histamine, or an adenosine derivative. The depressor effect of successive saliva samples decreased; saliva secreted in response to sympathetic nerve stimulation caused a greater depressor effect than parasympathetic saliva. Although Feldberg and Guimarais studied many of the properties of the depressor substance, they were unable to identify it.

Werle and von Roden (1935,1939) suggested that the hypotensive properties of saliva and salivary gland extracts were due to the presence of kallikrein, a substance which had also been found in the pancreatic juice. By comparing some chemical and pharmacological properties of the salivary hypotensive substance with those of kallikrein, Werle and von Roden established with reasonable certainty that the depressor effect of saliva was due, not to acetycholine as Secker believed, but to kallikrein. An investigation of the kallikrein content of salivary glands of various species (man, pig, dog, cattle and cat) showed that, in all cases, the submaxillary gland contained the highest concentration. It is of interest that kallikrein was not found in any of the salivary glands of the horse. Werle and von Roden were unable to explain the significance of the presence of kallikrein in salivary glands. The occurrence of kallikrein in human saliva and parotid gland was confirmed by Koryani, Szeres and Hatz (1937).

b) Properties of salivary kallikrein

Kallikreins, a group of enzymes with powerful pharmacological actions, are present in an active and/or inactive (but activatable) form in many glandular organs. Frey and his co-workers (1928,1930) coined the name kallikrein (Greek, kallikreas = pancreas) because they believed that the pancreas was the source of kallikrein found in salivary glands, blood, urine, etc. However, more recent work leads to the conclusion that the pancreas is not the sole source of kallikrein, and that the kallikreins from different sources are similar, but not identical, molecules. Thus, kallikreins from different sources differ in their molecular weights, (Frey, Kraut and Werle, 1968), immunological specificities (Webster, Emmart, Turner, Moriya and Pierce, 1963), electrophoretic mobilities (Moriya, Pierce and Webster, 1963) and their susceptibilities to various inhibitors (Webster and Pierce, 1961,1963).

Salivary kallikrein has been isolated and highly purified (Werle and Trautschold, 1963; Moriya, Moriwaki et al 1966). Moriya et al used a crude acetone powder of human saliva as a starting material; by adsorption on DEAE-cellulose, acetone fractionation, and gel filtration on Sephadex they produced a fraction with a purification factor of X2300. However, even this highly purified fraction was not homogeneous as shown by cyanogum electrophoretic analysis.

Werle and his co-workers (1967,1968) have described the amino acid composition of kallikrein from hog submaxillary gland. This kallikrein is a glycoprotein, containing glucosamine and galactosamine and with a molecular weight of 32,800.

Table 1

Composition of Hog Submaxillary Gland Kallikrein

	mole/mole
Lys	16
His	10
Arg	4
Asp	32
Thr	18
Ser	23
Glu	25
Pro	21
Gly	23
Ala	11
Cys½	8
Val	22
Meth	2
Ileu	14
Leu	24
Tyr	5
Phe	6
Try	5
Glucosamine	6
Galactosamine	3
Hexoses	0

Ekfors et al (1967) have described four isozymic forms of a peptidase resembling kallikrein in rat submaxillary gland.

Although a few instances have been reported where kallikrein might act directly, in most cases all kallikreins exert their effects by the release of kinins from a substrate in plasma or lymph, a reaction analogous to the release of angiotensin by the action of renin on angiotensinogen. The only known natural substrate for kallikreins is kininogen, an α_2-globulin. It is thought that there are two different kininogens in plasma: a low molecular weight form (approximately 50,000) and a high molecular weight form (over 100,000), (Jacobsen 1966a,b; Pierce 1968). The kallikreins are highly specific enzymes, hence the kallikreins from one mammal do not release kinin from the substrates of other mammals. For example, cat salivary kallikrein fails to release kinin from the substrate in horse plasma (Bhoola, Morley, Schachter and Smaje 1965).

As mentioned previously, kallikrein concentration is highest in the submaxillary gland; the amount increases with age, but is not influenced by changes in diet (Werle 1960). Chronic parasympathetic (Chorda tympani) denervation of the submaxillary gland greatly reduces the depressor activity of saliva and gland extracts; sympathetic denervation, on the other hand, does not significantly affect the depressor activity of saliva (Emmelin and

Henriksson 1953). Ligation of Wharton's duct for about three days also causes a marked reduction in salivary kallikrein in rabbits (Mattioli and Mattioli 1947a,b), rats, mice and dogs (Werle, Vogel and Lentrodt 1960; Werle and Trautschold 1963) and cats (Beilenson, Schachter and Smaje 1965,1968). This disappearance of kallikrein after duct ligation suggests that the enzyme has a exocrine function.

Emmelin and Henriksson (1953) suggested that salivary kallikrein may be localized within the demilune cells of the salivary gland: chronic parasympathetic denervation is associated with a fall in kallikrein content and a marked reduction in the size of the demilune cells. Sympathetic denervation, on the other hand, does not produce any marked structural changes in the gland, nor any significant change in depressor activity.

More recently the subcellular localization of salivary kallikrein has been investigated by Bhoola (1966,1968,1969) and by Erdos et al (1968) using a combination of differential and density gradient centrifugation. The results show that kallikrein in the submaxillary glands of various species (rat, guinea pig, cat, rabbit and dog) is located in granules which sediment in the nuclear and heavy mitochondrial fractions; these granules are in serous acinar cells and are probably zymogen granules with a diameter of approximately 1-2 μ.

c) Possible physiological role of salivary kallikrein

Ungar and Parrot (1936) quickly followed up the discovery of the presence of kallikrein in saliva (Werle and von Roden, 1936) by suggesting that the enzyme was the mediator of atropine-resistant functional vasodilatation induced by parasympathetic nerve stimulation in the submaxillary gland. To understand the significance of this suggestion it is necessary to describe the controversy over the postulated existence of vasodilator nerves.

In 1858 Claude Bernard presented evidence for the existence of both vasoconstrictor and vasodilator nerves to the submaxillary gland of the dog: after cutting the sympathetic trunk, the venous blood from the gland became brighter red and the velocity of flow increased. Stimulation of the peripheral end of the sympathetic nerve produced a decreased velocity of blood flow, while stimulation of the chorda tympani nerve or vinegar in the dog's mouth produced a marked increase in flow rate. The idea that both the secretory and vasodilator effects of chorda tympani nerve stimulation are mediated by acetylcholine is thrown into doubt by the observation of Heidenhain (1872) that whereas the former is abolished by atropine as expected, the latter is not significantly reduced.

An explanation for this anomaly was given by Barcroft and his co-workers (1912,1914). Barcroft measured oxygen consumption of the secreting submaxillary gland before and after administration of atropine. He found that whereas

salivation was abolished after atropine, the increased oxygen consumption and the vasodilator effect were both unaffected. He concluded that the increase in blood flow which occurred on chorda tympani nerve stimulation was in some way secondary to the increase in metabolic activity of the gland. However, Barcroft did not make any suggestions concerning the chemical link between increased metabolism and vasodilatation, and it was not until 1936 that Ungar and Parrot published the idea that kallikrein was the mediator of atropine-resistant chorda-induced vasodilatation in the submaxillary gland.

This idea fell into abeyance for about 20 years until it was upheld and extended by the work of Hilton and Lewis (Gautvik, Hilton and Torres 1968, Hilton 1960, 1962, 1963, 1970; Hilton and Lewis 1955a,b, 1956, 1957; Hilton and Torres 1967; Lewis 1960, 1962, 1963, 1967). These workers maintain that during secretory activity kallikrein passes from the acinar cells to the interstitial fluid, where it releases a vasodilator peptide (kinin) from an α_2-globulin substrate. This hypothesis would obviate the necessity of postulating the existence of vasodilator nerve fibers, but it does not explain why the secretory action of acetylcholine is atropine-sensitive, whilst the kallikrein-releasing action is atropine-resistant.

The most recent evidence brought forward by Hilton to support this idea involves measuring the concentration of the kallikrein substrate in venous blood before, during,

and after chorda tympani nerve stimulation. There are two substrates for kallikrein in plasma (Jacobsen 1966a,b): substrate 1 is acted on by plasma kallikrein, whilst substrate 2 is acted on exclusively by glandular kallikreins (Gautvik, unpublished). It was found that substrate 2 was reduced by up to 60% during and immediately after glandular activity whilst substrate 1 was unaffected (Gautvik et al 1968). It would be interesting to study the effects of sympathetic nerve stimulation and post-atropine parasympathetic nerve stimulation on substrate depletion. The fact that substrate depletion occurs at a time when the blood flow is increased does not necessarily mean that the two are linked.

The conclusions of Hilton and Lewis have been challenged by Schachter and his co-workers on the following grounds:

1) After desensitization of the glandular blood vessels to bradykinin the vasodilatation produced by chorda tympani nerve stimulation was relatively unaffected (Bhoola, Morley, Schachter and Smaje, 1965).

2) "Normal" chorda-induced vasodilatation can be produced in glands in which the substrate for kallikrein is absent, and in glands which have been depleted of kallikrein by chronic duct ligation and subsequent sympathetic nerve stimulation (Bhoola et al 1965; Beilenson, Schachter and Smaje, 1965,1968).

3) Sympathetic nerve stimulation is more effective than parasympathetic in liberating kallikrein into the saliva; however the main vascular effect of the sympathetic is vasoconstriction (Beilenson et al, 1965,1968).

4) There is a close parallel between the curves relating secretion rate of saliva or increase in blood flow at different frequencies of chorda tympani nerve stimulation; however, the curves of output and concentration of kallikrein in the saliva are distinctly different from the others. For example, the vasodilator response is almost maximal when the concentration of kallikrein in the saliva is almost minimal (Beilenson et al, 1968).

The conclusion that salivary kallikrein is unlikely to play a significant role in the vasodilatation which accompanies parasympathetic nerve stimulation is supported by the work of Webster (Skinner and Webster, 1968): the vascular effects of bradykinin injected close-arterially are abolished by carboxypeptidase B, whereas the vasodilatation produced by chorda nerve stimulation is unaffected.

Histochemical and electronmicroscopical techniques have been used to demonstrate that the blood vessels of the submaxillary gland of the cat are innervated by both parasympathetic and sympathetic nerves (Garrett, 1966a,b). Garrett believes 'that it is reasonable to assume that such parasympathetic "fibers" to blood vessels are vasodilatory'.

If it is finally shown with reasonable certainty that there are parasympathetic cholinergic vasodilator nerves to the submaxillary gland, then one is still left with the question of the function of salivary kallikrein, and as yet there does not seem to be a satisfactory answer.

2. Hypertensive substances in saliva and salivary glands.

a) Historical survey

Several workers have noted that the depressor response to intravenous injection of saliva was preceded by a brief and variable pressor effect. Secker (1934a) thought that the rise in blood pressure was due to the nicotinic action of acetylcholine, since it was accentuated after atropine. Larson (1936) discounted the idea that acetylcholine was the pressor agent in saliva on the grounds that protein-free ultrafiltrates of saliva were without effect on the blood pressure; this suggested that the pressor effect was due to a substance of high molecular weight. Feldberg and Guimarais (1935) showed that if a submaxillary gland had been subjected to prolonged sympathetic nerve stimulation, saliva collected during subsequent parasympathetic nerve stimulation had very low depressor activity, but still showed marked pressor activity. Further, the blood vessels of the dog were insensitive to the pressor agent in saliva from the submaxillary gland of the cat or dog, whereas a pressor response was obtained on injection of cat, dog or human saliva into the cat. The pressor agent was shown to

be thermolabile and atropine-resistant, but was not identified.

A hypertensive substance has been extracted from dog submaxillary gland (Werle and von Roden, 1939). The glands were ground with sand in the cold, then an aqueous extract made. The first extract showed mainly depressor activity, whereas a second extract showed mainly pressor activity when tested on the blood pressure of the dog. The active substance was said to be different from any other known pressor agent, and was shown to have the following properties: non-dializable, thermolabile, inactivated on incubation with serum, precipitated by alcohol or acetone, action not blocked by atropine, no action on isolated guinea-pig ileum. The pressor agent differed from renin and from vasopressin in that its action was very brief and did not diminish when a second injection was given. Werle did not find a pressor agent in the parotid glands of the dog, nor in the submaxillary glands of cats and cattle.

b) Iso-enzyme of renin

A hypertensive substance has also been found in submaxillary glands of white mice (Werle, Vogel and Goldel, 1957; Turrian, 1960; Werle, Baumeister and Schmal, 1962; Werle, Trautschold and Schmal, 1963). Aqueous extracts of the glands caused a long lasting (30 - 60 minutes) rise in blood pressure of the dog, rat and mouse on intravenous injection. The pressor agent has been partially purified;

it is an isoenzyme of renin which releases angiotensin I and II from a plasma substrate. Mice on a high salt diet or receiving subcutaneous injections of desoxycorticosterone acetate have a decreased concentration of renal renin, whereas submaxillary 'renin' is unaffected (Turrian, 1960). Oliver and Gross (1967) showed that the hypertensive agent in mouse submaxillary gland is located in the striated cells of the excretory ducts; the concentration increases on androgen administration. Three-day duct ligation caused a fall in enzyme concentration in parallel with a fall in the content of excretory granules in the striated cells. It was concluded that the renin-like substance is associated with some exocrine function of the submaxillary gland, although its specific function remains unknown.

According to Werle et al (1957) a hypertensive renin-like substance was found only in the submaxillary glands of the white mouse. Submaxillary glands of other animals (including rat, guinea pig, dog, cat, cow and sheep) were devoid of pressor activity. However, he did note that submaxillary gland extracts from the dog, pig, cow and horse caused a small, brief rise in blood pressure.

c) Hypertensive effect of bradykinin

Kinins released from a plasma substrate by the enzyme kallikrein are usually considered as hypotensive peptides. However, it should be noted that under certain conditions bradykinin produces a hypertensive effect on

intravenous injection (Croxatto and Belmar, 1961; Lecomte, Troquet and Dresse, 1961; Lecomte, Cession-Fossion and Troquet, 1963; Lecomte, Troquet and Cession-Fossion, 1964; Lang and Pearson, 1968). The usual depressor effect of bradykinin is replaced by a pressor response in rats with an arterial blood pressure below normal (eg. after treatment with the ganglion-blocking agent pentolinium tartrate, or 8 - 24 hours after nephrectomy). The fact that the hypertensive effect of bradykinin is seen at low doses (0.005 - 0.1 µg/100gm) must be kept in mind when utilizing the blood pressure assay for mixtures of bradykinin and hypertensive substances.

Intravenous or intracarotid injection of bradykinin or kallidin in cats produces a biphasic response: a depressor effect followed by a pressor effect. The latter was reduced or abolished after adrenalectomy or ganglion blockage, and was absent in spinal or decerebrate cats (Lang and Pearson, 1968). It was concluded that the pressor response to bradykinin and kallidin is mediated by sympathetic mechanisms and involves a central component.

d) Sialotonin

The study of the pressor agent in cat submaxillary saliva has been extended by Moriwaki, Beilenson and Schachter (1968); the pressor agent was named SIALOTONIN. Sialotonin produces a brief (5 - 15 sec) but marked rise in blood pressure on intravenous injection into the cat. This

response was not significantly affected by treatment of the test animal with atropine (500 µg/kg), phenoxybenzamine (500 µg/kg), hexamethonium (1 mg/kg) or carboxypeptidase B (3-4 mg/kg), but was abolished if the saliva were first incubated with trypsin or chymotrypsin (2.5 mg/ml saliva, 37°C, 30 min.). Sialotonin is thermolabile, non-dialyzable and is unaffected by kallikrein inhibitors. Whereas kallikrein in submaxillary saliva is secreted in much higher concentrations during sympathetic nerve stimulation, sialotonin is secreted only during parasympathetic nerve stimulation. Moriwaki et al separated kallikrein and sialotonin by gel filtration of cat submaxillary saliva on Sephadex G-100; sialotonin was eluted in earlier fractions than kallikrein, suggesting that it has a larger molecular size. It was concluded that sialotonin is a large molecular compound, probably a protein, which differs from other known pressor agents. These workers were unable to prepare a stable, freeze-dried preparation of sialotonin, therefore further investigations were hampered.

3. Objectives of present experiments

1. To separate kallikrein and sialotonin and make a stable preparation of the latter.

2. To investigate the effects of sialotonin on regional blood flow. Preliminary experiments by Moriwaki et al (1968) indicated that partially purified sialotonin has a very

marked vasoconstrictor action on the blood vessels of the small intestine; the studies failed to demonstrate a constrictor action of sialotonin on the blood vessels of the submaxillary gland itself.

Chapter II

METHODS

1. Animals and anesthesia

Cats (approximately 3 kg. and of either sex) were deprived of food about 18 hours before an experiment. Drinking water was available ad libitum. Anesthesia was induced with chloroform in a closed box, then maintained with ether on a face mark until a cannula was inserted for intravenous injection of chloralose (50-80 mg/kg). A tracheal cannula was inserted so that artificial respiration could be given if necessary.

2. Collection of saliva

The excretory duct of the submaxillary gland (Wharton's duct) was exposed through an incision in the midline of the lower jaw. The duct was cannulated with a fine glass cannula, about 5 mm rostral to the point at which the duct was crossed by the chorda lingual nerve.

The parasympathetic nerve supply to the submaxillary gland runs in the chorda tympani nerve, a branch of the chorda lingual nerve. The latter was exposed, cut near its point of exit from the skull, and a tie placed on the distal end. The sympathetic nerve was exposed in the neck region and separated from the vagus nerve. Both sympathetic and parasympathetic nerves were mounted on bipolar platinum electrodes and immersed in warm paraffin.

The nerves were stimulated supramaximally with square waves of 0.4 msec duration and at a frequency of 20/sec. The parasympathetic nerve was stimulated continuously for several minutes at a time, whereas the sympathetic nerve was stimulated for 20 second periods separated by 20 second rest intervals. (Prolonged sympathetic nerve stimulation causes vasconstriction and hence cessation of salivation.) Saliva was collected in small, weighed, glass bottles and was either used on the same day as it was collected or was stored at 4°C or -20°C.

3. Method for assay of kallikrein and sialotonin

Blood pressure was measured from a femoral artery using a Statham P23D pressure transducer and recorded on a Grass Polygraph or a Sanborn Medical Recorder. Heparin was injected at the start of an experiment (10 mg/kg i.v.). Saliva was usually diluted 1:10 and injected into a femoral vein (volume, 0.2 - 0.4 ml., followed by 0.4 ml. saline wash). The height of the pressor response was considered indicative of the sialotonin content of the saliva; the depressor response was used to measure the kallikrein content. This method gave extremely approximate estimates of kallikrein and sialotonin content of saliva.

4. Gel filtration of saliva

Saliva from the cat submaxillary gland, diluted 1:2 in 0.15 M NaCl was applied to a column of Sephadex G-150 (1.5 x 20 cm.) and eluted with 0.15 M NaCl (pH 8.2) at

4°C. The flow rate was 1 drop/7 seconds. Fractions (2 ml) were collected and their absorbancy at 280 mμ measured with a Beckman DU Spectrophotometer. The fractions were assayed for kallikrein and sialotonin the same day; in a few exceptions the fractions were frozen or freeze-dried.

5. Measurement of regional blood flows

a) Methods

Venous outflow. In a few early experiments blood flow through the submaxillary gland and the hind limb of the cat was measured with a drop-counter using the open circuit venous outflow method, as described by Bhoola, Morley, Schachter and Smaje (1965). This method was not used in later experiments because the time constant was too slow.

Electromagnetic flowmeter. The flowmeter used in these experiments was a Nycotron type 372 which measures both pulsatile and mean arterial flow. (Range, 5 ml/min upwards, depending on the sensitivity of the probe.) The flow probe used in each experiment was chosen so that its diameter was slightly smaller than that of the blood vessel, thus ensuring a close fit. The flowmeter was calibrated using a calibration factor given with each probe.

Thermal dilution flowmeter. The flowmeter has as its sensing element a thermistor, metallic oxide semiconductor, the temperature of which is kept at about five degrees above the ambient temperature of the blood. Both the sensor and the reference thermistor are mounted in the same glass

tube so that they are in the flow stream in order to minimize thermal lag. The flow of the fluid past the sensing element cools it by forced convection. A negative feedback system maintains the temperature of the sensor constant, and the fluctuations of the D.C. signal which drives the RF amplitude modulator, is a function of velocity. The RF amplitude modulator provides the heating power for the thermistor.

$$E = Kf(v)$$

where K is a constant depending on the fluid

E is the DC voltage

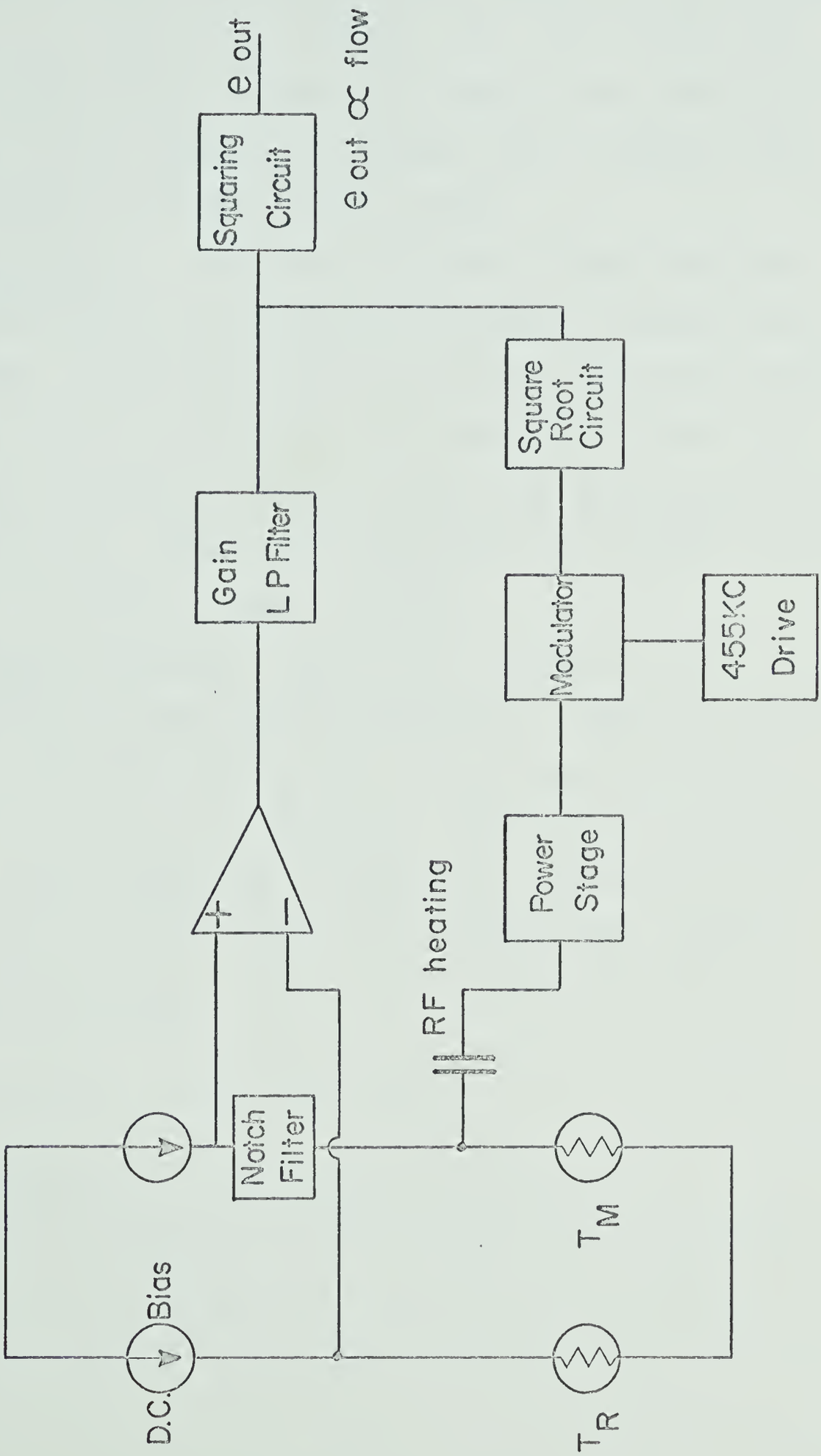
v is the velocity of the fluid at a distance away from the sensor.

This function has been shown to be $f(v) = \sqrt{v}$ (Karpinski, 1970). Calibration of the instrument is then carried out using plasma or saline as the fluid and a calibrated syringe driver. Linearization circuits in the flowmeter have been set up so that in the range .05 cc/min. to 150 cc/min. the voltage is directly proportional to the velocity of the fluid, $E = Kv$ and since the cross-sectional area of the glass tube is known. $E = KQ$ where Q is the volume flow rate in cc/min. The reference thermistor compensates for the small differences in blood temperature. Frequency response of the flow measuring system is 120 Hz.

b) Regional blood flows

Submaxillary gland. Blood flow through the

THERMO DILUTION FLOWMETER BLOCK DIAGRAM



submaxillary gland was measured using a thermodilution flowmeter, which was placed in the external jugular vein about 2 - 4 cm. from the gland. All veins (except the submaxillary vein) entering the jugular vein were ligated. Samples to be tested were injected into a femoral vein or retrogradely into the lingual artery.

Hind limb. Blood flow through a femoral vein was measured using a thermodilution flowmeter inserted into the vein. Small branches of the femoral vein were ligated except for one branch which was cannulated so that the flowmeter could be flushed through with heparinized saline if necessary. Samples were injected into a small branch of the ipsilateral femoral artery.

Small intestine. Blood flow through the small intestine was measured using an electromagnetic flowmeter. A flow probe was placed around the superior mesenteric artery as close to the aorta as possible. The inferior mesenteric artery was ligated and a cannula was inserted into an anastomosing branch between the inferior and superior mesenteric artery.

Chapter III

RESULTS

1. Vasoactive substances in saliva

Saliva secreted from the submaxillary gland of the cat during parasympathetic nerve stimulation (chorda saliva) caused a biphasic change in arterial blood pressure on intravenous injection into the same cat. The usual dose of chorda saliva (0.02 ml, diluted 1:10 in saline to give an injection volume of 0.2 ml) produced a sharp increase in blood pressure (20 - 25 mm Hg) which lasted for 5 - 15 sec. This was followed by a more prolonged (25 - 30 sec.) fall in blood pressure, known to be due to kallikrein. Saliva secreted during sympathetic nerve stimulation did not produce an initial pressor response, but only a very pronounced depressor effect (Fig. 2).

Saliva from dogs, rabbits, rats, guinea pigs and humans does not contain significant amounts of a pressor substance (Moriwaki, Beilenson, Schachter, unpublished), therefore the source of sialotonin used in the present experiments was saliva secreted from the cat submaxillary gland during parasympathetic nerve stimulation.

2. Separation of kallikrein and sialotonin

A satisfactory separation of kallikrein and sialotonin was obtained by gel filtration of chorda saliva on Sephadex G-150. A typical example of the protein elution pattern is shown

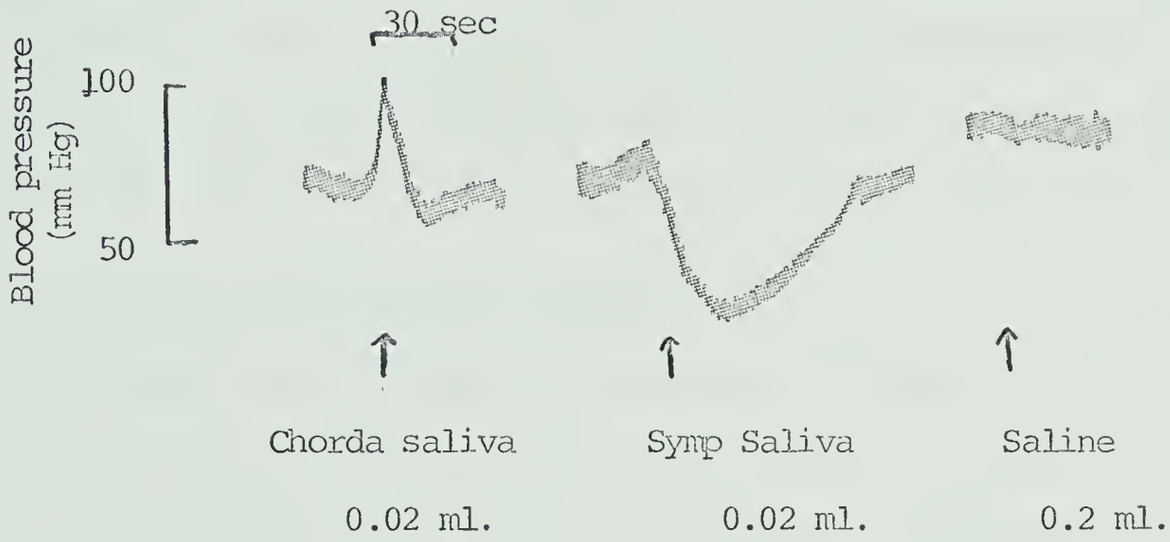


Fig. 2 Effect of intravenous injection of Chorda saliva and Sympathetic saliva on cat arterial blood pressure

in Fig. 3a, together with the results of the assay of some of the fractions on the cat blood pressure (Fig. 3b). The elution pattern shows two definite protein peaks, but only samples from the first peak were vasoactive: fraction 8 contained sialotonin, fractions 11, 12 possibly contain both sialotonin and kallikrein, although not definitely shown here, and fraction 17 contained kallikrein.

The pressor response to sialotonin was related in a linear fashion to the log of the dose injected (Fig. 4a & b). It was shown that 1 ml of chorda saliva yielded about 8 mg of freeze-dried powder; after gel filtration 1 ml of chorda saliva yielded 2.4 mg of freeze-dried "sialotonin fractions".

3. Attempts to stabilize sialotonin

The depressor effect of cat chorda saliva is still present after saliva has been lyophilized or stored at -20°C for several weeks (or even months). Unfortunately, the pressor effect of saliva is easily lost. For example, saliva which was known to contain sialotonin was stored overnight at 4°C or -20°C; when tested the following day the pressor effect was usually small or absent, even when the test animal was responsive to its own saliva. The loss of pressor activity was very erratic and unpredictable: in a few experiments pressor activity was undiminished after saliva had been stored at 4°C or -20°C for 2 weeks or lyophilized, whereas in most experiments the pressor activity was lost completely. It thus became imperative to find

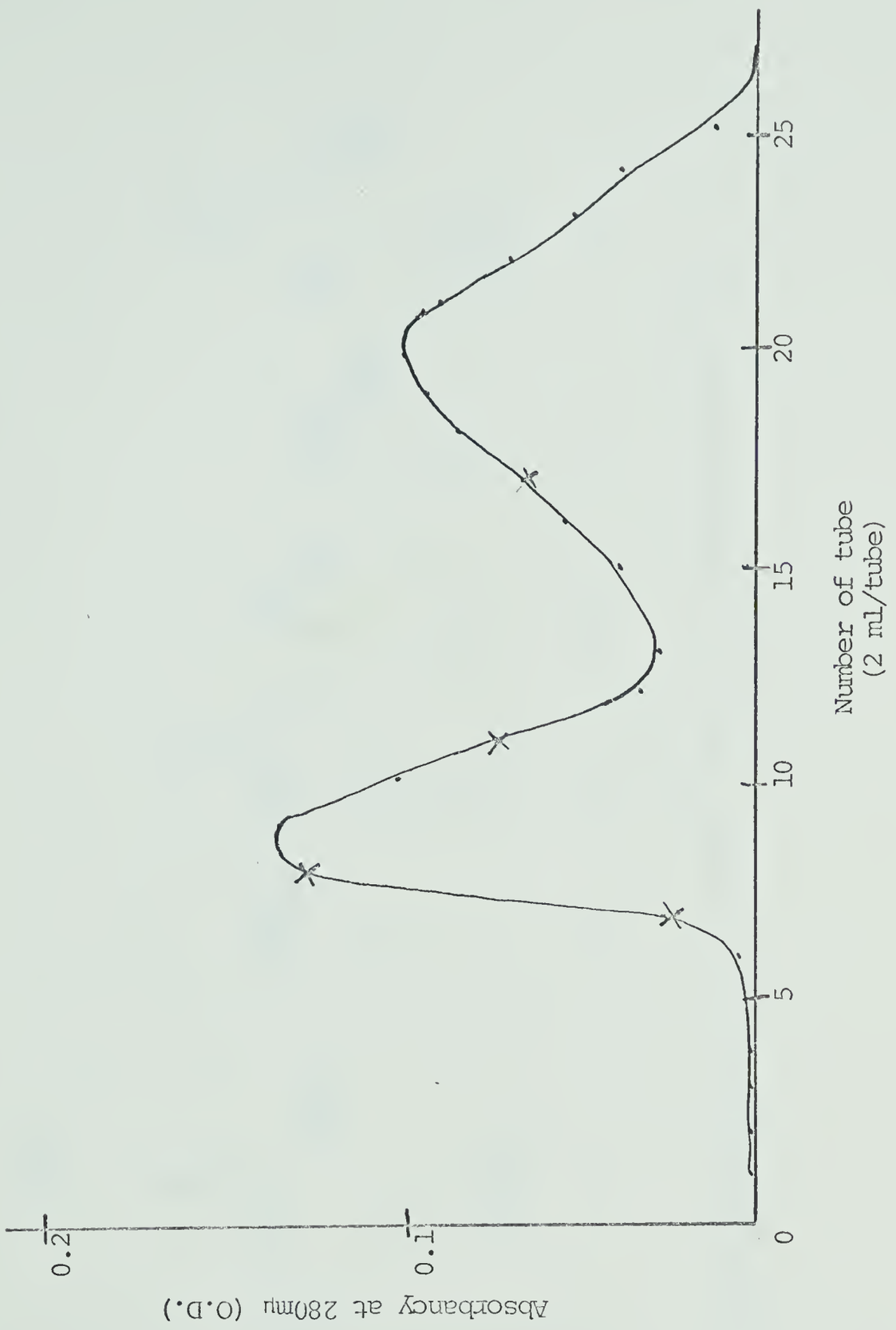


Fig. 3a Gel filtration (Sephadex G-150) of cat Chorda saliva
Optical densities of successive fractions
(X= samples were taken for blood pressure assay, Fig 3b)

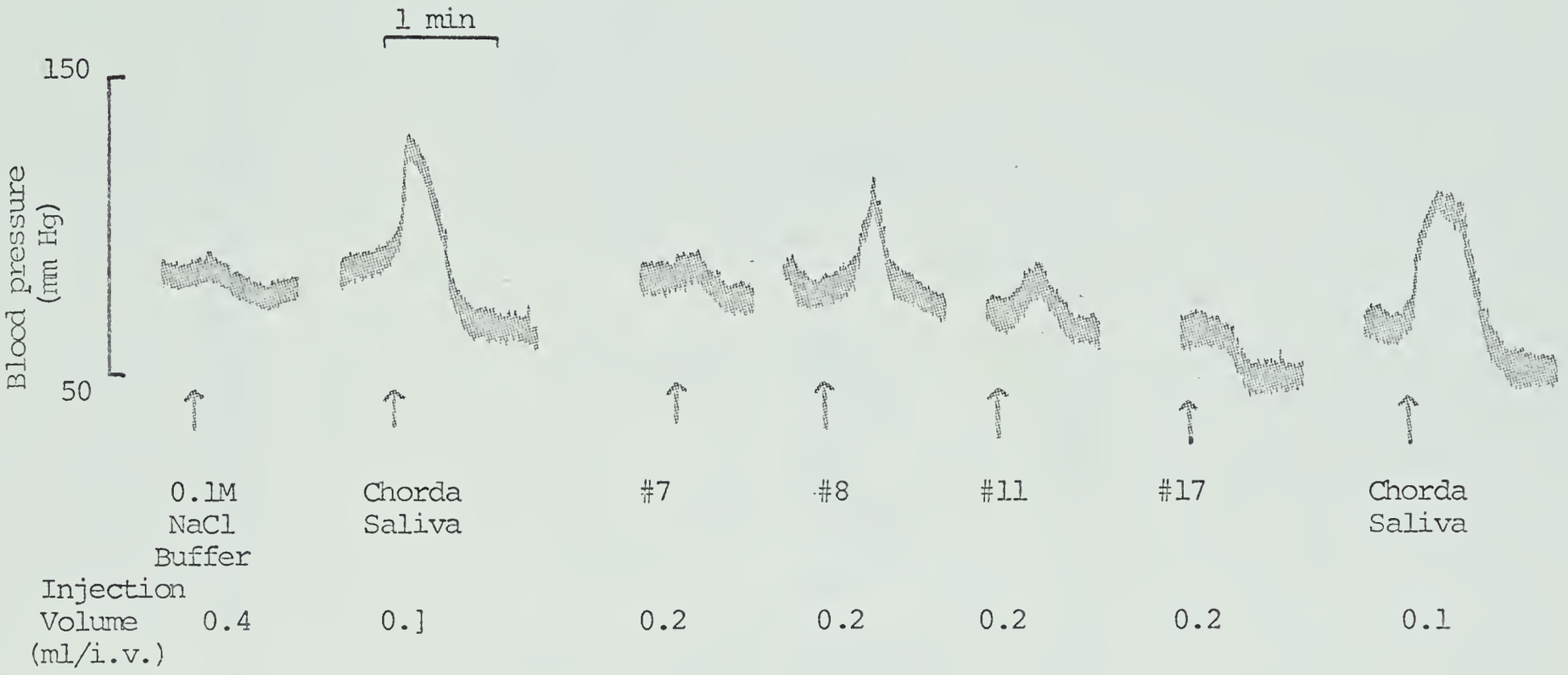
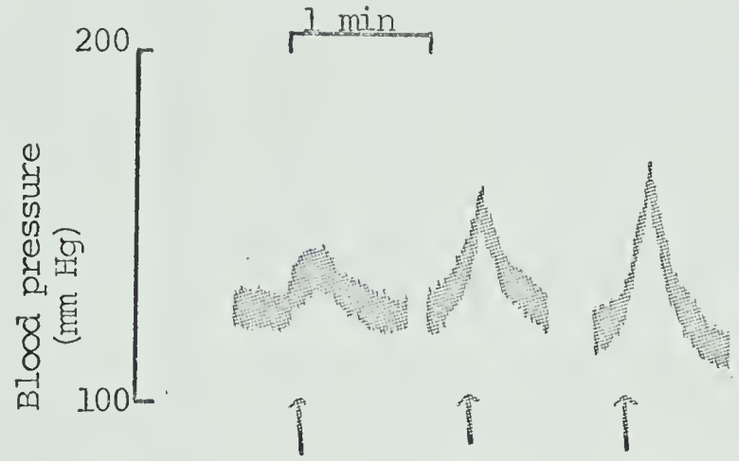


Fig. 3b Response of cat blood pressure to intravenous injection of fractions eluted from Sephadex G-150 column (see fig. 3a)



Dose of freeze-dried Sialotonin	200μg	400μg	800μg
Injection vol. (ml)	0.1	0.2	0.4

Fig. 4a Effect of intravenous injection of Sialotonin fractions on cat arterial blood pressure

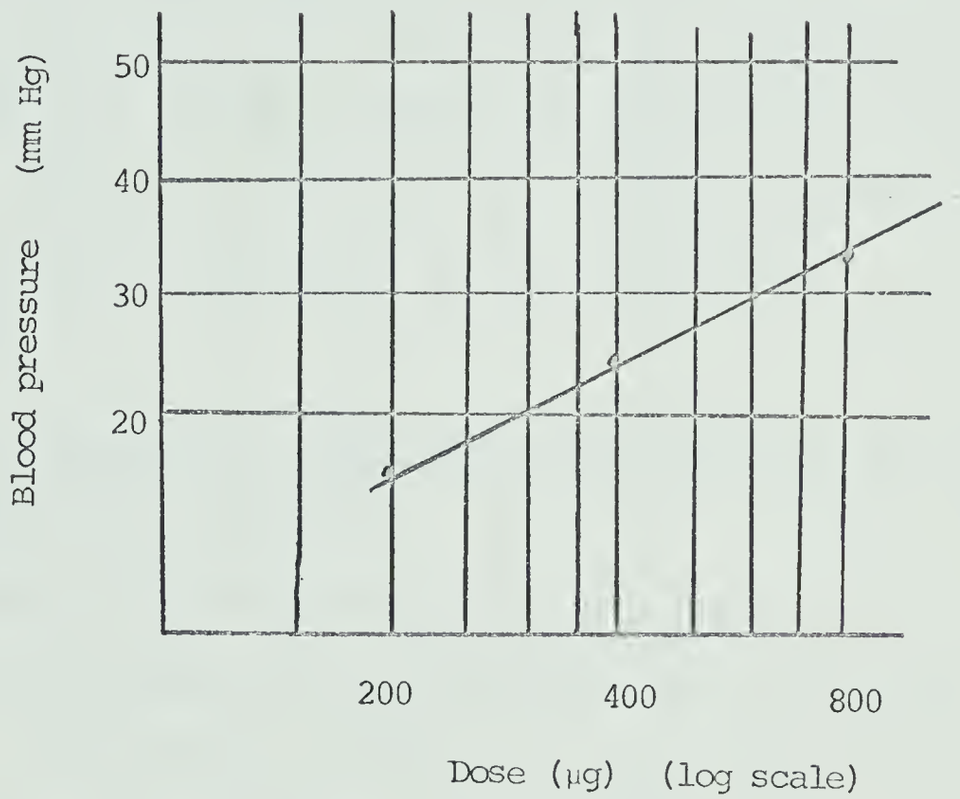


Fig. 4b Effect of intravenous injection of Sialotonin fractions on cat arterial blood pressure

a means of stabilizing sialotonin so that a stock source could be accumulated for future experiments.

Cat chorda saliva or sialotonin fractions eluted from a Sephadex G-150 column were lyophilized in the cold in the presence of 10^{-4}M $CaCl_2$.

As shown in Fig. 5 sialotonin could sometimes be at least partially stabilized by this procedure. However, the results were still unpredictable and it was concluded that more must be known about the composition and structure of sialotonin before a suitable method for stabilization can be selected. This of course sets up a vicious circle, since in order to purify a substance, it first has to be stabilized.....

4. Effect of sialotonin on regional blood flow in the cat

a. Submaxillary gland

Blood flow through the submaxillary gland was measured using a thermodilution flowmeter in an external jugular vein. Intravenous injection of cat chorda saliva caused a brief decrease in blood flow, followed by a slightly more prolonged increase in flow. These changes in blood flow were synchronous with the increase and decrease, respectively, of arterial blood pressure. As shown in Fig. 6 the basal flow through the cat submaxillary gland was .09 ml/min; chorda saliva (0.02 ml i.v.) caused a decrease to zero flow.

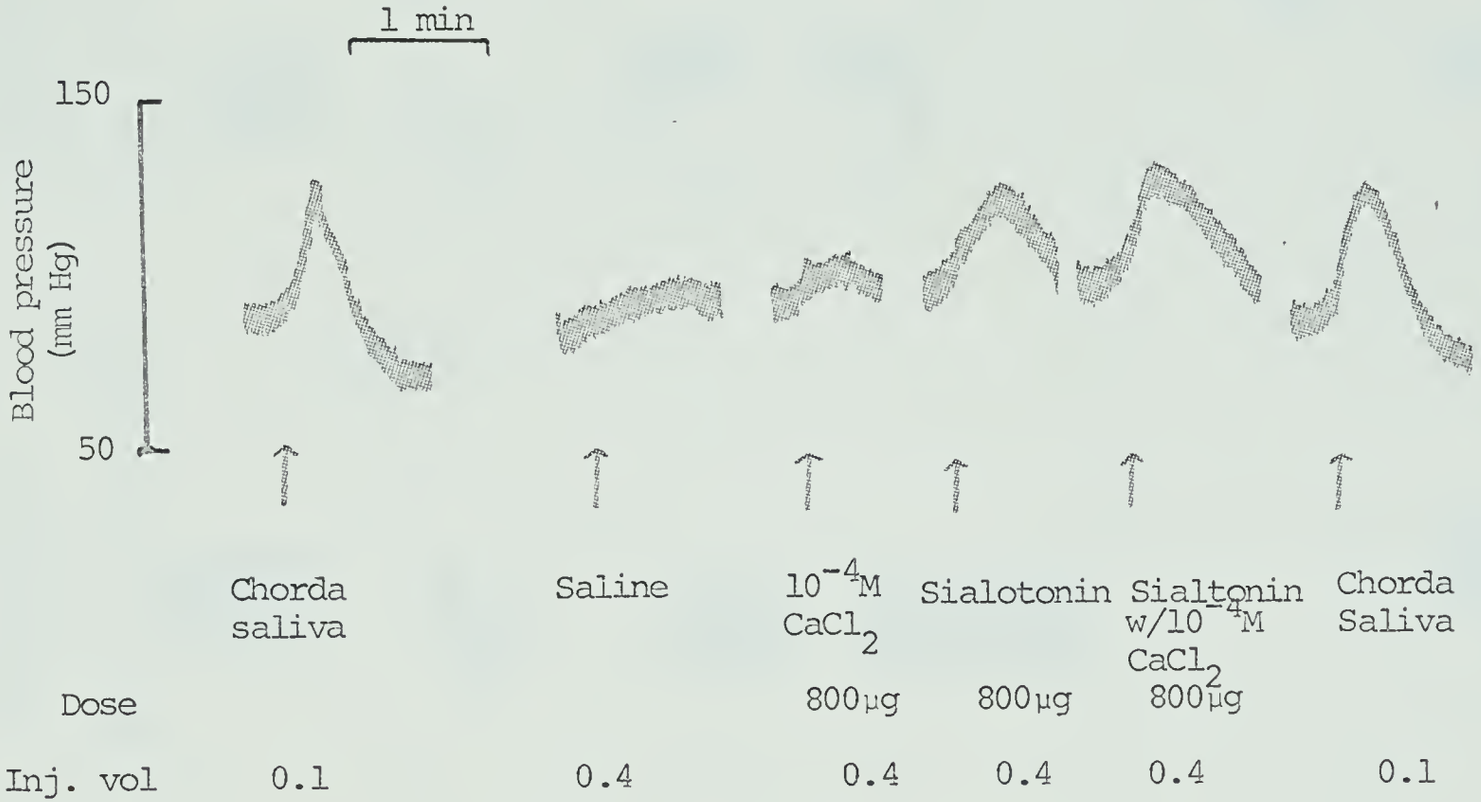


Fig .5 Attempted Stabilization of Sialotonin : Effects of Ca^{++}
(Sample - 200µg in 0.1 ml. of saline

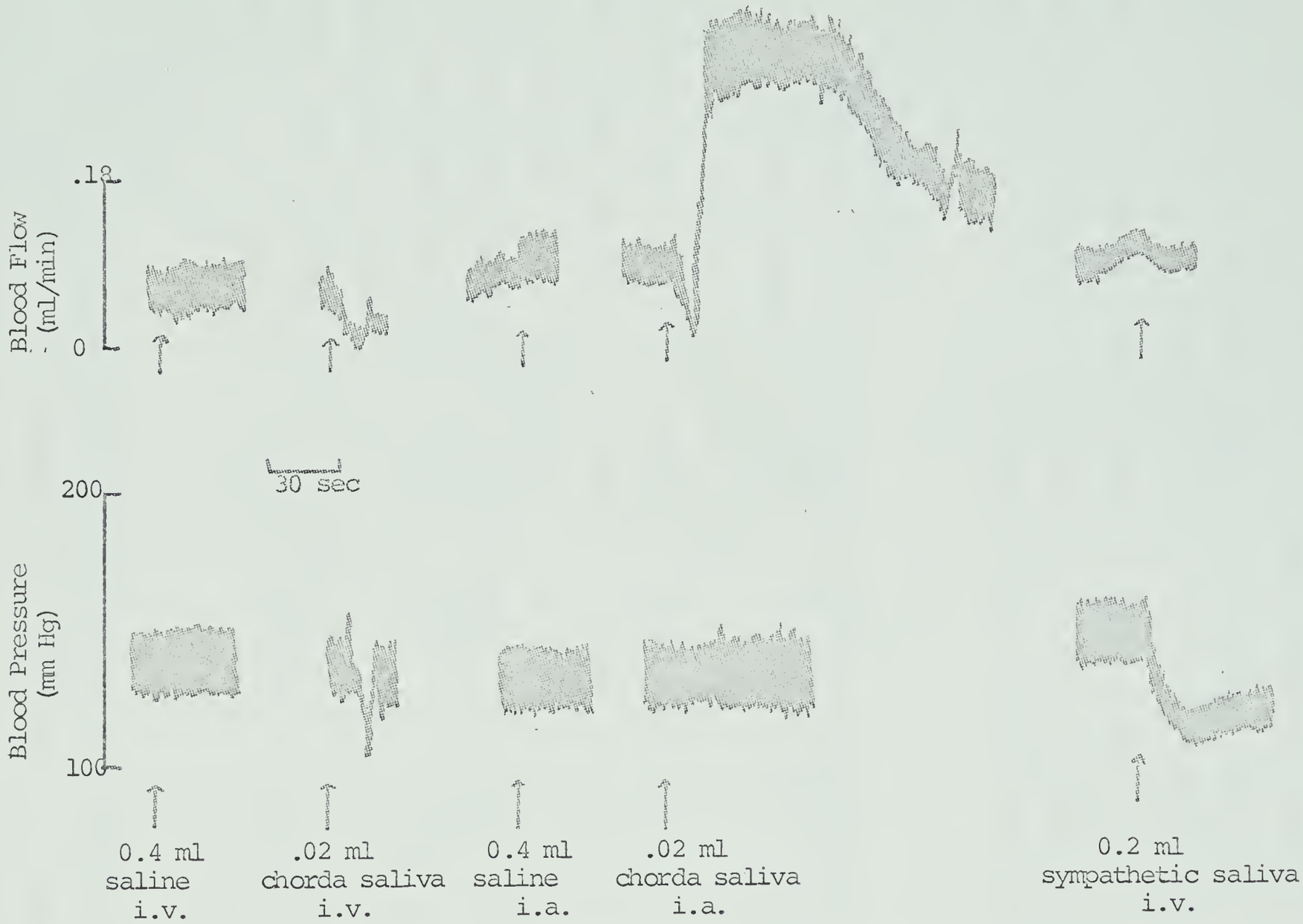


Fig 6 Effect of i.v. and i.a. injection of chorda and sympathetic saliva on cat arterial blood pressure and blood flow through the submaxillary gland of the cat

Injection of chorda saliva close-arterially usually did not affect the blood pressure but in most cases did cause an initial decrease, followed by a more pronounced increase in venous outflow from the gland (Fig. 7). Injection of sympathetic saliva (i.v. or i.a.) caused an increase in blood flow, associated with a marked fall in blood pressure.

b. Small intestine

Blood flow through the small intestine was measured using an electromagnetic flowmeter, with the probe placed around the superior mesenteric artery. Injection of chorda saliva, close arterially, caused a slight rise in blood flow (from 10 ml/min to 14 ml/min) followed by a fall (to 8 ml/min)(Fig. 8). The changes in blood flow thus appeared to follow the changes in blood pressure. This result is in disagreement with previous work which showed that a decreased blood flow through the small intestine coincided with a rise in blood pressure, suggesting that the former might be contributing to the latter (Moriwaki, Beilenson, Schachter, 1968).

c. Hind limb

Blood flow through a hind limb of the cat was measured using a thermodilution flowmeter in a femoral vein. Basal flow rates were approximately 0.5 - 0.9 ml/min. (It should be noted that basal flow rates measured using an electromagnetic flowmeter with the probe around a femoral artery were about 16 ml/min.)

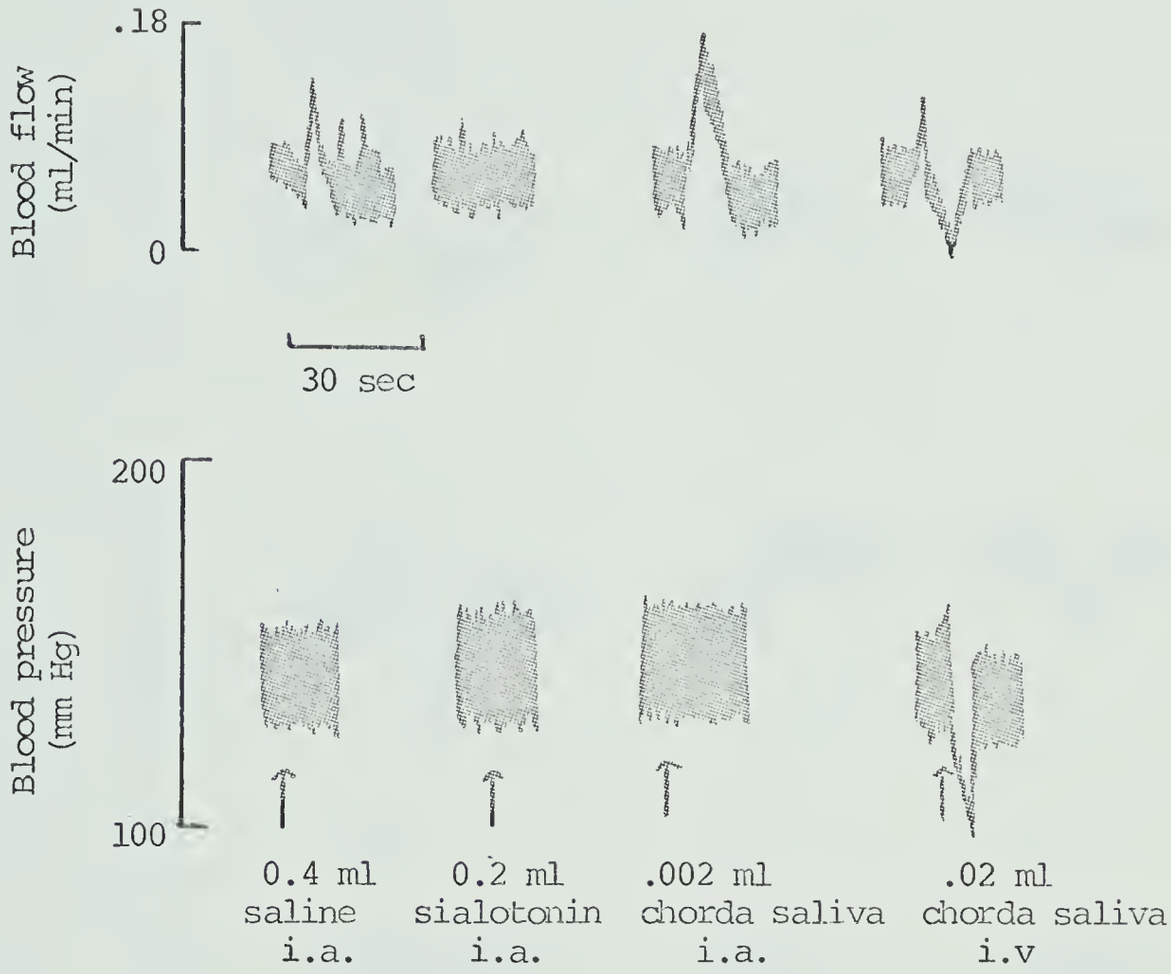


Fig. 7 Effect of i.v. and i.a. injecton of chorda saliva and sialotonin on arterial blood pressure and blood flow through the submaxillary gland of the cat

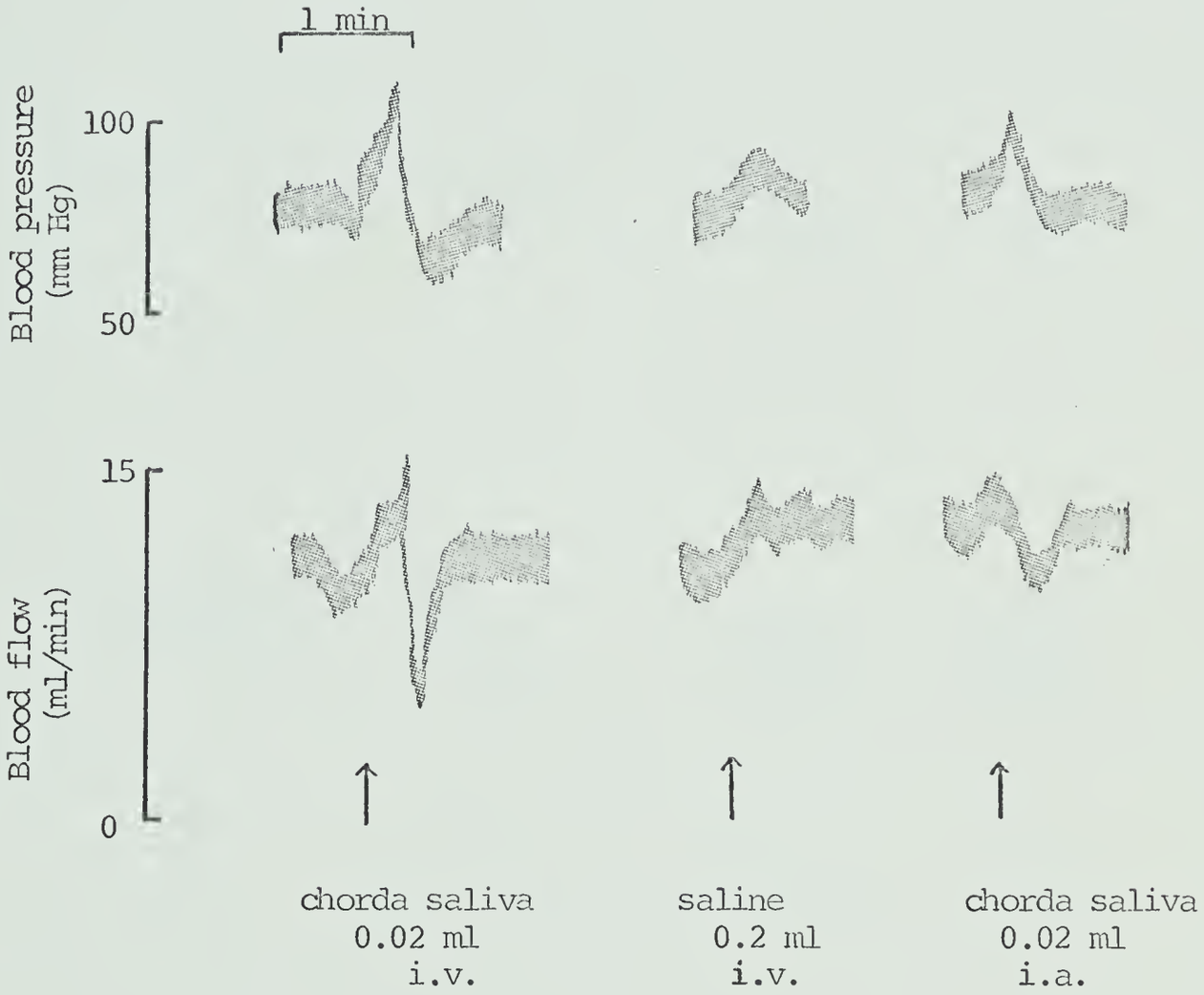


Fig. 8 Effect of i.v. and i.a. injection of cat chorda saliva on arterial blood pressure and blood flow through the small intestine (SMA) of the cat.

Intravenous injection of chorda saliva caused the characteristic biphasic response of blood pressure, but there was either no change in blood flow or a slight increase (Fig. 9). Close arterial injections of saliva or sialotonin fractions also failed to cause a significant decrease in hind limb flow.

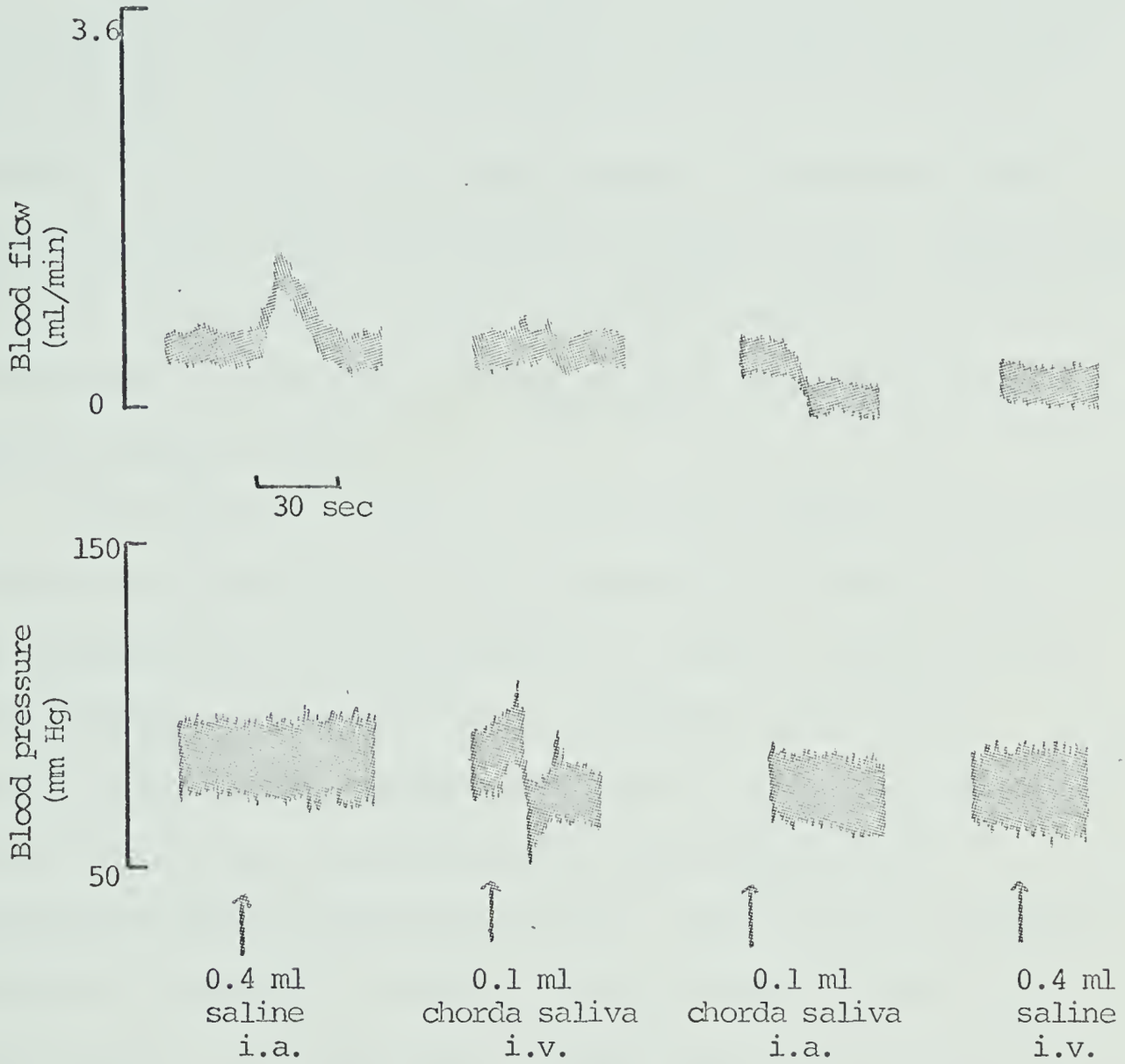


Fig. 9 Effect of i.a. and i.v. injection of cat chorda saliva on arterial blood pressure and blood flow through a hind limb of a cat.

Chapter IV

DISCUSSION

The properties and actions of the depressor substance in saliva, kallikrein, have been investigated thoroughly. In contrast, the substance responsible for the pressor effect, sialotonin, has been ignored. Sialotonin can be distinguished from low molecular weight pressor substances such as noradrenaline and angiotensin because it is non-dialyzable; it can be distinguished from renin by its very brief pressor action.

Sialotonin is found in appreciable amounts only in the saliva secreted from the cat submaxillary gland during parasympathetic nerve stimulation. Thus, sialotonin (a vasoconstrictor substance) is released only by the vasodilator nerve, whereas kallikrein (a vasodilator agent) is released more effectively by the predominantly vasoconstrictor sympathetic nerve. This finding suggests that the vasoactive substances in salivary glands do not play a part in regulation of blood flow during secretory activity. The significance of the differential release of kallikrein and sialotonin is still unknown. Since sialotonin has a very limited distribution this could mean either that its significance is small, or that it has a highly specialized role, the nature of which has not yet been discovered.

Kallikrein and sialotonin were separated by gel filtration on G-150 Sephadex, sialotonin being eluted in earlier fractions than kallikrein. Sephadex G-100 had been used in earlier experiments (Moriwaki, Beilenson, Schachter, 1968), but a better separation was obtained in the present work. From the elution pattern of sialotonin it was estimated that its molecular weight is greater than 100,000 (Porath, 1960; Gellotte, 1960). Sialotonin is probably a protein since it is thermolabile, non-dializable, and is inactivated by incubation with trypsin or chymotrypsin. Recent work by Ueki (personal communication) suggests that sialotonin is a glycoprotein: cellulose acetate electrophoresis of sialotonin fractions and staining with a Schiff reagent were used. There is of course a possibility that the sialotonin fractions were contaminated with kallikrein, which is known to be a glycoprotein. The evidence to support the idea that sialotonin is a glycoprotein is at this stage too inconclusive to allow for any categorical statements.

The chief difficulty in studying sialotonin is that it is so easily inactivated by lyophilization or storage at 20°C. Lyophilization in the presence of $CaCl_2$ was used in an attempt to prepare stable sialotonin. This method was partially successful in some, but not all, experiments, and better methods are still required for the stabilization

of sialotonin. Fresh saliva, tested in the same cat from which it was collected, was used in most experiments to ensure that active sialotonin was present.

The assay system for sialotonin (i.e. pressor effect) is neither very sensitive nor specific. It was hoped that an assay system based on measurement of regional blood flows could be developed.

In earlier experiments Moriwaki et al (1968) concluded that "our studies failed to demonstrate a marked constrictor action of sialotonin on the blood vessels in the salivary gland itself". Blood flow was measured by the open circuit venous outflow method using a drop counter. This method of blood flow measurement is too slow to record small rapid changes in blood flow, (time constant is dependent on drop size, frequency response from D.C. to 1 cycle). In the present experiments a thermodilution flowmeter was used (time constant = .013 sec., frequency response = 120 Hz). Contrary to earlier results it was shown that sialotonin does cause a brief vasoconstriction, coinciding with the rise in arterial pressure.

The advantages of the thermodilution flowmeter are:

a. It is easy to install in the animal

b. There is no necessity for an extracorporeal circulation

c. The time constant is very short, enabling transient changes in blood flow to be measured

d. The sensitivity range of the flowmeter depends on the tube diameter of the probe

e. It is easy to calibrate (flow is directly proportional to electrical output).

The main disadvantage of this flowmeter is the tendency for blood clots to form within the probe tube, even in well heparinized animals. In several experiments the venous drainage from the submaxillary gland or hind limb had to be blocked temporarily while the probe tube was removed and flushed through with saline.

The present experiments showed that injection of sialotonin caused a fall in blood flow through the superior mesenteric artery which was synchronous with a fall in arterial blood pressure, suggesting a passive change in blood flow. Earlier experiments (Moriwaki, et al, 1968) had shown that injection of partially purified sialotonin or of cat's chorda saliva reduced blood flow through the superior mesenteric artery by as much as 80% at a time when the arterial blood pressure was <u>increased</u>, suggesting that sialotonin has a highly specific vasoconstrictor action on the blood vessels of the small intestine. The reason for the discrepancy between the results is unknown.

Experiments on blood flow through a hind limb of the cat failed to show a vasoconstrictor action of sialotonin. In some experiments a slight increase in flow was seen: this might have been a volume effect or might have been due to contamination with kallikrein. The reason for the low-basal flow rate in most instances was assumed to be caused by a complete or nearly complete shutdown of the

vascular bed. It possibly could be a type of an auto-regulatory device similar to actions of other vascular beds.

Since relatively gentle handling of the intestines, or handling or removal of the kidneys have been shown to decrease or abolish the pressor effect of sialotonin (Moriwaki et al, 1968) it would seem desirable to pursue measurements of regional blood flows in greater detail in an attempt to determine the site(s) of action of sialotonin.

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